# QUANTITATIVE STUDIES ON THE ISLETS OF LANGERHANS

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ALMQVIST & WIKSELLS BOKTRYCKERI AB

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# Quantitative Studies on the Islets of Langerhans

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R. UNIV.
BIBLIOTHEEK
LEIDEN

The present communication comprises a survey of the following papers:

I. Hellman, B.: Methodological aspects on the differential cell count of the islet tissue in the rat. Acta Path. et Microbiol. Scandinav. 45, 336 (1959).
II. Hellman, B.: The total volume of the pancreatic islet tissue at different ages of the rat. Acta Path. et Microbiol. Scandinav. 47, 35 (1959).
III. Hellman, B.: The effect of ageing on the number of the islets of Langerhans in the rat. Acta Endocrinol. 32, 78 (1959).
IV. Hellman, B.: The volumetric distribution of the pancreatic islet tissue in young and old rats. Acta Endocrinol. 31, 91 (1959).
V. Hellman, B.: A method for rapid estimation of the islet volume in the rat pancreas based on counting instead of measuring the islet section surfaces. Acta Path. et Microbiol. Scandinav. 47, 21 (1959).
VI. Hellman, B. and Hellerström, C.: Diurnal changes in the function of the pancreatic islets of rats as indicated by nuclear size in the islet cells. Acta Endocrinol. 31, 267 (1959).
VII. Hellman, B.: The effect of protracted administration of small doses of alloxan on the islets of Langerhans in rats fed on diets rich and poor in carbohydrate. Acta Soc. Med. Upsal. 64, 393 (1959).
VIII. Hellman, B.: The frequency distribution of the number and volume of the islets of Langerhans in Man. 1. Studies on non-diabetic adults. Acta Soc. Med. Upsal. 64, 432 (1959).

Reference to these papers will be made with the Roman figures given above.

## Introduction

The central position of insulin as regards the etiology and treatment of diabetes makes it important to have as much information as possible about the endocrine organ which is responsible for the production of the hormone, i.e. the islets of Langerhans. In recent years a further reason for this has emerged from investigations which have indicated that the islets also appear to produce glucagon, a blood sugar raising factor of importance in liver glycogenolysis.

When Paul Langerhans (31) in 1869 at the age of 22 years described for the first time the cell conglomerations in the pancreas, which later were to bear his name, he did not express any opinion about their physiological rôle. The discovery twenty years later by Mehring and Minkowski (37) that pancreatectomy resulted in diabetes, demonstrated that the pancreas also played a part in carbohydrate metabolism. On the basis of histological observations, especially the profuse vascularisation in the islets, Laguesse (30) assumed in 1893 that the endocrine function was a property of the islet cells. The classical experiments which in 1922 led to the successful extraction of insulin (2) provided the conclusive evidence of the important endocrine rôle of the islets.

It is now certain that the islets of Langerhans consist in all mammals of at least two different cell systems, the A cells and the B cells. A convincing proof that it is the B cells which produce insulin was obtained by the finding that they could be selectively destroyed by alloxan. A certain amount of caution is still warranted as regards conclusions concerning the physiological significance of the A cells. Most investigations however support the concept that they are the production site for glucagon. Excellent reviews of the chemical and physiological properties of glucagon and of the evidence for and against its localization in the A cells have been written by Foa, Galansino and Pozza (7) and Lukens (35).

In our investigations on the structure of the islet organ and its changes under different conditions, special attention has been paid to the fact that it holds an exceptional position among the endocrine organs in the three following respects:

1) It is not a single distinct anatomical unit, but is dispersed in the form of different sized bodies within the exocrine parenchyma.

2) There are good grounds for assuming that functionally it is not a single endocrine organ, but is the production site for two substances, insulin and glucagon, with different effects on carbohydrate metabolism.

3) Its venous effluent discharges into the portal circulation, and thus the concentrations of insulin and glucagon are proportionately high in the liver.

## Plan of Investigation

An attempt has been made to solve certain methodological problems of essential importance in the quantitative histological analysis of the islets of Langerhans. Up to the present, determinations of the total volume of the islets have, because of the dispersion of the islet organ into different sized cell conglomerates, been particularly time consuming and necessitated the measurement of a large number of islets or islet section surfaces (V). The fact that the islets are composed of the A and B cells, which are also from a functional point of view non-equivalent, makes it desirable to complement the total islet volume determinations with differential cell counts and separate estimates of the total volumes of the two cell systems. As the methods previously employed are subject to systematic errors, which in the case of the rat are considerable, special attention has also been devoted to these problems (I, VII).

The dispersion of the islet organ into rather small constituents of different sizes raises the question of whether the distribution of the numbers and volumes of the islets into different size classes is characterized by any definite pattern. This has been estimated in rats of different ages by using the formulae derived by Wicksell (43, 44) for the relation between the observed distribution of spherical and ellipsoidal bodies in a section and their actual distribution in the organ (IV, VII). The results thus obtained encouraged the extension of these investigations to include also the islet organ in man. The first stage of such a series of investigations, consisting of analyses of the regional size frequency distribution from adult non-diabetics, is included (VIII). The post natal development has further been followed in the rat, both as regards the distribution of the total islet volume into different islet sizes (IV) and the values for the total volume and number of the islets (II, III). It has been shown that the composition of the diet is a factor which influences the total volume of the islet organ. Since the islets are composed of two different cell structures, each of which presumably produces its own blood sugar regulating factor, it seemed desirable to study also the effects of changes in the diet on the B/A cell number ratio and the total volumes of each of these two cell systems (VII). In addition to these latter investigations, another physiological aspect of normal A and B cells in the rat was studied by following the diurnal changes in their nuclear sizes (VI).

In recent years several authors have suggested that small quantities of alloxan, especially if administered repeatedly, stimulate the insulin producing cell structures. As the possible existence of such an excitatory effect on the B cells must be regarded as important, our earlier investigations (20) on the results of single sub-diabetogenic alloxan doses

have been supplemented with quantitative studies of the effect of a protracted administration of small alloxan doses on the islet organ in rats which were fed on diets either rich in, or poor in, carbohydrate (VII).

Details of the investigations mentioned above, together with discussion of the results, will be presented under the following three main headings:

A) Contributions to methods for the quantitative investigation of the islet organ with special reference to the rat. B) The normal structure of the islet organ and its changes under different physiological conditions. C) The effect of protracted administration of small doses of alloxan on the islet organ in the rat.

## A. Contributions to Methods for the Quantitative Investigation of the Islet Organ with Special Reference to the Rat

### *The Differential Cell Counts of the Islet Cells (I)*

The procedure for differential counts of islet cells from silver impregnated frozen sections has been studied in detail by Hultquist, Dahlén and Helander (26). It was shown that the ratio between the numbers of cells varied considerably in the individual islet section surfaces but became relatively constant if a sufficient number of cells were counted. With the recent development of satisfactory granule stains, most workers have made differential cell counts on thin paraffin sections, without taking into account certain potentialities of the method which can considerably increase its accuracy and scope. It was therefore considered advisable to put the usual mode of procedure for the estimation of the B/A cell number ratio in thin paraffin sections to a critical methodological test.

The characteristic nucleo-cytoplasmic volume ratios of the A and B cells and their position within the islet facilitated an evaluation of differential stains in the rat. The best results were obtained with Gomori's chrome-hematoxylin phloxine stain, which has therefore been used in the subsequent analyses. In attempts to impregnate the whole pancreas with silver according to a modification of Bielschowsky's technique, no silver impregnated cells in the usual meaning of the term, i.e. with a black cytoplasm, were obtained. It should however be noted that the nuclei of the A cells, after a suitable impregnation time, were characterised by a deeper rust brown tinge.

In the differential cell counts it was found best to use wide field optics with a total magnification of 500×. The generally accepted principle of only counting cells which contain whole nuclei or parts

of nuclei means that the cells with the largest cell nuclei are numerically over-represented. According to Floderus (6) the relation between the counted number of nuclei and the actual number per volume unit may be expressed as:—

$$n = N\,(a + 2R - 2h) \qquad (1)$$

$N$ = the number of cell nuclei with centres within the volume unit.
$n$ = the number of counted nuclei (all nuclear sections).
$a$ = the section thickness in $\mu$.
$R$ = the radius of the nucleus in $\mu$.
$h$ = the height in $\mu$ of the smallest nuclear fragments, that were counted.

It is thus evident that if the thickness of the section is kept constant, the number of nuclei counted must increase with a larger nuclear diameter; not only does the factor $R$ increase but $h$ also tends to decrease. Since the B cell nuclei in the rat are larger than those of the A cells, it follows that too high values are obtained for the B/A cell number ratio. In order to avoid such systematic errors it is therefore necessary to correct the original differential cell count to allow for the difference between the nuclear sizes. It must further be observed that the magnitude of the correction factor introduced in this way is not constant, but that it depends, apart from the section thickness, on the functional state of the islet cells and the age of the animals. The mean value of the systematic error in the B/A cell number ratio at different ages for the case in which the B cells were not subjected to any special stimulation was thus estimated for 4 $\mu$ thick sections, from a total of 60 rats, to vary between 4.3–10.6%, with the highest value in the new born and the lowest in the oldest, i.e. the 480 days old animals (15).

According to equation (1), a decrease in the systematic error of the original differential cell count would be expected as a result of an increase in the section thickness. That this was not the case was due to the fact that it was more difficult to identify the small nuclear fragments of the A cells. For example, in one pancreas doubling of the section thickness from 4 $\mu$ to 8 $\mu$ was accompanied by an approximate doubling of the $h$ value for the A cells, while the corresponding value for the B cells remained almost unchanged. The systematic error for the B/A cell number ratio thus increased from 5 to 12%.

It seemed advisable to make the differential cell counts in the form of double determinations, where each single determination included at least 1000 cells from at least 25 islets. In a total of 60 animals an average error of $\pm 7.88\%$ was obtained for the single determination, the highest value $\pm 11.36\%$ being observed in a group of 12 rats 480 days old, and the lowest $\pm 5.83\%$ in a similar number of new born rats.

Hess (22) introduced a graphical treatment of the results of differential cell counts, which has been subsequently used for islet studies in several species including the rat (40). According to this method the islet section surfaces were grouped with respect to the B/A cell number ratio, and the frequency in each group was plotted in the form of a histogram. Whether this diagram gives any biologically important information seems to be doubtful, as it is completely dominated by the small islet section surfaces. However, by classifying instead, the islet section surfaces with respect to the number of cells they contain and then calculating the B/A cell number ratio in each size class, certain conclusions can be drawn as to how the actual size of the islets influences the numerical ratio between the two islet cell types. If the B/A cell number ratio were the same in large and small islets, the peripheral position of the A cells in the rat would result in a much lower quotient for the small islet section surfaces, as these are composed to a great extent of peripheral sections of larger islets. Studies of the islet section surfaces from 12 adult male rats actually yielded however the opposite result, from which it is inferred that the relative frequency of B cells is very high in the small islets. Our subsequent investigations (15) have confirmed that the relative overrepresentation of the B cells in the small islets is characteristic of both young as well as old rats, but that it becomes more pronounced with increasing age.

## *The Estimation of the Total Islet Volume (V)*

The methods described in the literature for the determination of the total volume of the islets of Langerhans are particularly time consuming, since a large number of islets or islet section surfaces must be measured or at least classified into different sizes. Moreover the islet volume shows great individual variations, and it is therefore often necessary to investigate a large number of experimental animals in order to evaluate statistically the effects of different factors.

Our investigations on the rat have shown that the distribution of the islets into different size classes is characterised by a definite pattern regardless of the age of the animals (IV, VII). The regularity of the actual islet size distribution raised the question as to whether merely counting the number of islet section surfaces exceeding a certain size would be sufficient to obtain an estimate of the total islet volume in the rat.

The relation between the number of islet section surfaces in a series of pancreatic sections, taken at regular intervals, and the total islet volume were studied in altogether 75 Wistar rats of different ages. It was shown that the lower size limit for the islet section surfaces

included could be placed so high that it corresponded to the area of a circle with a diameter of 109.4 $\mu$. Exclusion of the small islet section surfaces is justified by the form of both the numerical and volumetric distribution curves, and also means a considerable gain in time. The islet section surfaces included in the measurements will henceforth be described as 'the counted number of islets'.

If the animals were divided into three groups according to age, Group I consisting of new born and 5 and 21 days old animals, Group II 100 days old animals and Group III 480 days old animals, the islet volume in each such group constituted a linear function of 'the counted number of islets'. For each group, the regression equation, the correlation coefficient and the confidence limits for the islet volume, corresponding to a $P$ value of 0.05, were calculated. The value for the two first named factors were, in Group I, $r=0.993$ and $Y=0.0082\ X+0.0136$, in Group II, $r=0.989$ and $Y=0.0096\ X-0.1367$ and in Group III, $r=0.984$ and $Y=0.0112\ X-0.5919$.

The reproducibility of determinations of the total islet volume solely by counting the larger islet section surfaces was also studied in a total of altogether 56 rats of about 100 days old, in which it was known that the islets retained their regular size distribution, in spite of the fact that changes had occurred in the B/A cell number and volume ratios as a result of extreme variations in the carbohydrate content of the diet and the protracted administration of small amounts of alloxan (VII). A comparison made for each of the 56 animals between the values obtained by, (1) inserting the 'counted number of islets' in the regression equation given earlier for the 100 days old animals, and (2) a more complicated procedure in which the total surface area of the islet section surfaces was measured, showed the difference between the results of the two methods to be insignificant. The mean difference for all the animals was $-0.0054\pm0.0080$ mm$^3$.

Some mention should be made of certain practical aspects of the volume determinations, based on the principle that the total islet volume constitutes a linear function of the number of the large islet section surfaces. Where the experimental conditions are such that it may be suspected that the islet volume has deviated from its otherwise symmetrical size distribution, a check should be made beforehand to exclude this possibility. The histological treatment must be done in an absolutely uniform manner for all the pancreases during one experiment. A suitable procedure for which the regression equations given above are valid has been described elsewhere (II). The distance between the serial sections analysed can be varied according to the degree of accuracy required in the determinations. The equations given were calculated for a distance of 300 $\mu$. In order to determine quickly, during the systematic scanning of the sections, whether or not an islet should be included, it is convenient to insert into the focal plane

of the ocular a circle and ellipses of varying eccentricities, all the figures being equal in area and corresponding to the size of the minimal islet section area included in the measurements.

Although this method for determining the total islet volume has up to the present been applied only to rats, it seems probable that in principle it may also be applicable for quantitative islet analyses in other species.

### *Determinations of the Total Volumes of the A and B Cells Separately (VII)*

Since the A and B cells of the islets of Langerhans do not constitute any single functional unit, it is important to determine the total volumes of the two cell systems separately. The only attempts to do this previously seem to have been by Maclean and Ogilvie (36) and Gepts (8, 9). The total volumes of each of the two cell systems were obtained by these authors by combining the determinations of the total islet volume with the results of differential cell counts. In the case of the rat at least, criticism can be directed against the fact that the results of the original differential counts were not corrected for the over-representation of the B cells (see above) and that no attempt was made to determine the volume of the residual islet tissue, i.e. that which is not A or B cells. However of greater significance is the fact that the values obtained solely from the total islet volume and B/A cell number ratio are only correct provided that the individual mean cell volumes of the A and B cells are equal.

By determining the nucleo/cytoplasmic volume ratio by means of point sampling, and then combining these values with the results of caryometric determinations, the individual mean cell volumes for both the A and the B cells were calculated in altogether 40 adult rats. It was shown in this way that the individual mean cell volume for the B cells was in fact more than 3 times as large as for the A cells. As a consequence of this, the values for the total A cell volume, as given by Gepts (8) in his analyses of the effects of antidiabetogenic sulphonamides on the rat islets, are probably 2–3 times larger than the actual values, while the B cell volumes were calculated correspondingly altogether too low.

The necessity of taking into account the individual mean cell volumes for each of the two cell systems in connection with the calculations of the total A and B cell volumes separately is further emphasized by the fact that the mean cell volume is not constant but is subject to variations dependent on the functional state of the cells and age. In another connection (16) we have thus observed that the mean volumes of the single B cells in new born rats were more than 4 times as large as the A cells.

## B. The Normal Structure of the Islet Organ and its Changes under Different Physiological Conditions

### *The Frequency Distribution of the Islet Number and Volume in Rats of Different Ages (IV, VII)*

The relationship between the actual size distribution and the apparent distribution, i.e. that observed in a section, has been thoroughly investigated mathematically by Wicksell (43, 44). For the case in which an organ contains different sized, evenly distributed spherical or ellipsoidal bodies, he was able to demonstrate that although the apparent and actual distributions differ, there is a relationship between them which can be expressed by an Abelian integral equation. Approximations of these equations yield linear equation systems which can be used in calculating the actual distribution from that observed from the sections.

Wicksell's formulae were first applied to a specific biological problem in studies of the secondary follicles in the spleen (21). Tejning (41) later used the same equation system in work on the effect of the caloric composition of the diet on the islets of Langerhans in the rat. His most important finding was that the volumetric[1] islet distribution in 9 month old rats showed a remarkable degree of symmetry, while the corresponding numerical distribution curve was extremely asymmetrical.

In our studies of the post-natal development of the islets in the rat it has been considered of value to follow the growth of the islet organ also from the curves for the actual size distributions of the islets. These distributions were calculated by means of Wicksell's formulae, and those islet section surfaces in which the geometric mean of the largest and smallest diameter was less than 46.9 $\mu$ were excluded. In making the determinations Tejning's procedure was modified as follows:

1. In the histological treatment of the pancreas, all compression of the organ was avoided. This, as has been pointed out by Larsson (32), can be a source of error.

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[1] *Terminology.* The terms defined below apply only to the actual (estimated) and not to the apparent (observed) islet distributions.

1. *Islet diameter* refers to the diameter of a sphere which has the same volume as that of the actual islet.

2. *Numerical islet distribution* is the distribution of the number of islets among the different size classes, i. e. in terms of the magnitudes of the mean islet diameters of each class.

3. *Volumetric islet distribution* is the distribution of the islet volumes among the different size classes. The total islet volume in each class is the product of the number of islets in that class and the volume corresponding to the mean islet diameter.

2. Instead of carrying out measurements on 30 $\mu$ thick sections, sections 4 $\mu$ and 7 $\mu$ thick were used.
3. Serial sections which were used for islet analyses were taken with different intervals in young and old rats. Subsequently all values were corrected to an interval of 300 $\mu$, so that the constants given by Tejning could nevertheless be used to calculate the actual size distribution.
4. The identification and classification of the large number of islet section surfaces at a magnification of 240× was facilitated by a special microscopical arrangement which has been described in an earlier publication (14).
5. In drawing the apparent distribution curves, the differences in class width were taken into consideration.

Wicksell's formulae were derived for spherical or ellipsoidal bodies. As the islet section surfaces only in exceptional cases deviated markedly from a circular or elliptic shape, it seemed permissible to infer that the islet form satisfies Wicksell's requirements. In using the formulae it was further presupposed that the islets, regardless of their size, were distributed evenly within a layer, the thickness of which was varied in animals of different age and had a maximum value of 308 $\mu$ in the adult rats. This latter assumption is in fact an approximation, but introduces an error too small to be important in the calculation of the actual distribution curves.

The volumetric distributions of the islets were investigated in altogether 131 rats. In 75 of the rats the distributions at different ages were compared (IV), and the remaining rats were used in a study of the effects of small doses of alloxan (VII). In studying the effect of age, the animals were killed on the 1st, 5th, 21st, 100th or 480th days of life, while in the alloxan experiment all the animals were about 100 days old. Independent of the age, the islet volume was distributed according to a symmetrical curve. The main part of the islet volume was thus constituted by the medium sized islets. Tejning was able to show in his analyses of 9 month old rats that a larger total islet volume was in general connected with a shifting of the maximum of the curve towards the right, i.e. towards larger islets. It was evident in our curves that this tendency was to be found in all the ages investigated. If the mean distribution curves of the different age groups were compared, it was apparent that the increase of the islet volume, which occurred with ageing, was for the two oldest groups also accompanied by a shift of the peak of the curve to the right, and consequently also by a broadening of the curve.

If the mean volumetric distribution curves at different ages were expressed as cumulative percentages and then converted into logarithms, the values were grouped around straight lines up to a value corre-

sponding to 93–99% of the total islet volume. This shows that these curves, in the essential parts of their courses, can be regarded as closely approximating normal distributions. As has been pointed out in the study of the effects of small doses of alloxan (VII), the volumetric distribution curves must always deviate from a strictly normal form in their most extreme parts; in the paper on the volumetric islet distribution at different ages (IV), the word 'normal' is therefore used with the limitations mentioned.

As a consequence of the symmetrical volume distribution the corresponding numerical distribution curves were extremely asymmetrical, the number of islets progressively increasing with a decreasing islet diameter. In the 75 rats of ages varying from the new born to 480 days, the logarithms of the islet numbers for those size classes which contained the overwhelming majority of islets studied were found to lie on an approximately straight line (17). If it is assumed that the islet volumes are quite normally distributed it can be shown theoretically that the particular form of normal curve obtained in these experiments must result in such a linear relation between the logarithm of the islet number and islet diameter (size class). For the 56 rats in the alloxan experiment (VII), approximate linearity was found up to and including about the 10th size class.

To summarize, the analyses of the actual size distribution of the islets showed that, at all ages studied, the islet organ in Wistar rats was arranged in a definite pattern, and that thus its normal post-natal growth also conforms to a regular order.

## *The Frequency Distribution of the Islet Number and Volume in Adult Non-diabetic Man (VIII)*

Although there are some reports on the apparent size distribution of the islets of Langerhans in man (10, 11, 27), very little is known about the actual size distribution. The information available in the literature is limited to an incomplete analysis of the pancreas from a single individual, whose age and sex are not mentioned (42). The fact that the actual size distribution of the islets in the rat was, regardless of age, arranged in a characteristic pattern (see above), raised the question of whether the frequency distributions in other species also followed similar lines. As a more detailed knowledge of the human distribution curves would be of especial value, different aspects of this problem have been investigated. Only the results of distribution studies from different regions of the pancreas in adult non-diabetic persons are discussed here.

In man also, both the shape and dispersion of the islets within the exocrine parenchyma were suitable for the application of Wicksell's

formulae in the calculation of the actual islet distributions. The investigations were carried out partly on fresh pancreatic material (6 cases) obtained by resection of the tip of the tail of the pancreas during gastric operations, and partly on post mortem material which was taken between 5 and 20 hours after death. The main object of the study of the post mortem material was to compare different regions within one and the same organ, and thus necessitated analyses of a very large number of islets from each individual. The analysis was therefore limited to 5 organs. The distribution curves for each region investigated were calculated on the basis of at least 500 islet section surfaces, in which the geometric mean for the largest diameter and the diameter at right angles to it were $\geqq 46.9\ \mu$. The maximum number of such regional analyses made on any single pancreas was 12.

The numerical distribution curves were asymmetrical in all regions in such a way that the number of islets increased progressively with decreasing diameter. The majority of the volumetric distribution curves on the other hand had a characteristically symmetrical form. The total islet volume was in this way mainly composed of the medium sized islets, while the contributions of the largest and the smallest islets were proportionately rather small. In the tail region all the volume distribution curves were symmetrical. In the few cases in which a more obvious deviation from the basic symmetrical form was observed, these curves were from those parts of the head and body adjacent to each other. The reason for asymmetry in this particular region is not clear. A possible explanation may be that the area mentioned represents the fusion point for the ventral and dorsal pancreatic primordia.

The mean distribution curves for the different regions in each of three pancreases in which the analyses were carried out at regular space intervals do not completely correspond to the curves which describe the distributions of the total islet volume and the number of islets for the whole pancreas. This discrepancy which is due to the variations in both the size of the regions and the density of the islet population was at least in one case unimportant because of the mutual similarities of the regional curves. It was therefore of interest to establish that the mean distribution curves for the islet volumes were not only symmetrical but that a probit transformation showed that at least in two organs the curves fitted well with a normal distribution in the main part of their courses, i.e. with the exception of the extreme values. Another point of similarity to the rat was that the logarithms for the mean islet numbers were grouped around straight lines for those size classes which contained the overwhelming majority of the islets studied.

Since the pancreas is subject to early post mortem changes, comparison with fresh material was made to determine whether these

changes influenced the form of the curves. No effects were however noted. Neither was it possible to demonstrate any definite deviations in the form of the volume distribution curve for pieces of the pancreatic tail which were removed at autopsy 17 hours after death and then kept unfixed at a temperature of +4°C for up to 48 hours after the autopsy.

In the autopsy material both the actual numbers of islets and their total volumes in the different regions were expressed per volume unit of pancreatic parenchyma. No relation was found between the relative frequency of the islets and the form of the volume distribution curve in a region. If different parts within one and the same pancreas were compared, the tail showed in general the highest values for the relative number of islets, and therefore also for the relative islet volume. There were however large individual variations with regard to the proportions between one part and another, so that in one case the highest values were found in the body and the lowest in the tail. The mean value for the percentage volume of the islets for the three parts of the pancreas was on an average 1.31, with a highest value of 1.87 and a lowest of 0.91.

As mentioned above the actual size distribution of the islets in the new born rat was characterised by the same regular pattern as in the adult animals, but the maximum of the volume curve was displaced to the left. Further investigations confirmed that this also seems to be the case in man (18).

## *The Total Volume and Number of the Islets of Langerhans at Different Ages of the Rat (II, III)*

In any study of the post-natal growth of the islets of Langerhans in the rat, it is necessary to take into consideration the fact that apart from the age of the animals, many other factors such as the strain of the rat, the composition of the diet, the method and time of day of the killing etc. can influence the results. The studies of the total volume and number of the islets in different ages were carried out on altogether 75 male rats of the Wistar strain, which were killed by decapitation at 8 p.m. on the 1st, 5th, 21st, 100th or 480th days of life, i.e. at logarithmically fairly regular intervals. The animals were fed on a standard diet consisting of 38 cal.% carbohydrate, 24.5 cal.% fat and 37.5 cal.% protein. As an increase in the blood sugar in the mother during pregnancy can result in volume changes in the islet organ of the new born rats (1, 25), the defined diet was as a precaution given to the mother from approximately 14 days before mating.

The histological treatment of the pancreas was similar for all the age groups. The relative volume changes of the whole organ during

different stages of the histological procedure up to and including infiltration with paraffin were estimated by an application of Archimedes principle. The greatest changes were in molten paraffin, as in this medium a pronounced and continuous volume decrease was found, in contrast to the alcohols, where the main part of the volume reduction occurred initially. As a result of fixation in Bouin's solution neutral red intravitally accumulated in the islet cells was to a great part retained during the subsequent passage through alcohols, toluene and paraffin. In measuring islets stained in this way, before and after the histological procedure, no significant differences in shrinkage were found between young and old animals. It should however be noted that the measurements carried out in this way do not give an exact measure of the absolute islet shrinkage, but only a rough estimate of whether this is of the same order of size in the different age groups.

In the analyses, only those islets whose diameters were $\geq 46.9\ \mu$ were included. It was evident from the volumetric distribution curves that the islets thus not included only represented a rather unimportant part of the total islet volume. As an estimate of the so-called 'total number' of the islets is inexact, the introduction of such a lower size limit can be considered justified in making comparisons of the number of islets in different individuals.

Both the number of islets and their total volume increased considerably with increasing age. For the new born rats an average islet number of $243.1 \pm 21.9$ was obtained compared with $3319.8 \pm 148.1$ for the 480 days old animals, i.e. an increase of more than 13 times. The corresponding figures for the islet volume were $0.142 \pm 0.016$ mm$^3$ and $3.386 \pm 0.204$ mm$^3$, i.e. the value for the oldest animals was almost 24 times larger. As is seen from the standard errors (15 animals in each group), the individual variations were considerable within any one age group.

Characteristic differences were established between the post-natal growth of the islet organ and the increase in the pancreatic and liver weights. The logarithm for the weights of the last named organs thus constituted linear functions of the logarithm for the body weight in the age groups studied. The corresponding regression between the logarithms for the islet volume or the number of islets and the body weight were on the contrary curvilinear, so that the rate of increase was proportionately small between new born and 5 days old rats, while that between the two oldest age groups (100 and 480 days) was equal to or exceeded that of the liver and pancreatic weights. Since the initial rate of increase in the number and total volume of the islets was slow but accelerated with age, the ratio between either of these two quantities and either the body, liver or pancreatic weights was high at birth, later becoming reduced to a minimum value and subsequently rising again significantly between 100 and 480 days.

In each of the age groups a strongly positive correlation was obtained between the total volume and the number of the islets. Another consequence of the regular arrangement of the actual size distribution of the islets was that the actual number of islets could be regarded as a linear function of the number of apparent islet section surfaces exceeding a certain size. For the relation between the number of islet section surfaces with a diameter of $\geq 46.9\ \mu$ and the actual number of islets a correlation coefficient of 0.991 was obtained for the two youngest age groups taken together and of 0.990 for the two oldest. This relationship seems of importance with regard to the possibilities of rapid estimation of the actual number of islets, analogous with that which has been described for the total islet volume, i.e. by only counting the islet section surfaces which exceed a certain minimal size. In most of the age groups there was statistical support for a positive correlation between the number or total volume of the islets and the body weight. On the other hand no such correlation could be shown in relation to the liver or pancreatic weights, after their common positive correlation to the body weight had been eliminated by means of partial correlation calculation.

The reason for an accelerated increase in the total volume and number of the islets with increasing age is not clear. Analyses of the A and B cells separately showed that the increase in the total islet volume between 100 and 480 days was mainly contributed to by the B cells, although proportionately the increase in the A cells was greater (16). It may be postulated that ageing *per se* leads to an activation of diabetogenic factors, one of which may possibly be the increase in the A cells, leading to augmented demands on the B cells. On the other hand the great regenerative capacity of the rat B cells, clearly demonstrated by the spontaneous recovery which often occurs in alloxan diabetes (33) and the lack of success of attempts to produce permanent diabetes with hypophyseal extract (38, 45), suggest that the rat can compensate the growing influence of the diabetogenic factors with advancing age by an adequate increase in the number of B cells.

## *The Effect of the Caloric Composition of the Diet on the Islets of Langerhans in the Rat (VII)*

Since the caloric composition of the diet is considered an important factor not only as regards the treatment of diabetes but also in its etiology, it is of interest to study the relationship between the carbohydrate content of the diet and the islet morphology. Tejning (41) found that rats fed for a rather long period on a carbohydrate rich diet had a larger total islet volume than if the diet were poor in

carbohydrate. Since it seemed of interest to extend these observations, we have studied the influence of the carbohydrate content of the diet on the B/A cell number ratio and the volumes of each of the two cell systems.

Altogether 29 male rats were used, of which 14 were fed *ad lib* from about the age of 3 weeks on a diet extremely rich in carbohydrate (74 cal.% carbohydrate, 7 cal.% fat and 19 cal.% protein), and the remainder on a diet poor in carbohydrate (2 cal.% carbohydrate, 42 cal.% fat and 56 cal.% protein). As these rats also served as controls for an experiment on the effects of the protracted administration of small quantities of alloxan (see below), they all received repeated daily subcutaneous injections of Sørensen's phosphate buffer. The animals were killed at about the age of 100 days, by bleeding under ether anaesthesia.

In a comparison between the two groups there was no statistically significant difference between the total volumes of the islets. The value for the carbohydrate rich diet was $1.679 \pm 0.115$ mm$^3$ compared with $1.628 \pm 0.133$ mm$^3$ for the carbohydrate poor diet. The B/A cell number ratio differed on the other hand highly significantly. With the carbohydrate rich diet the ratio was $2.89 \pm 0.13$, and with the carbohydrate poor diet $2.15 \pm 0.08$ ($P < 0.001$). The separate volumes of the A and B cell systems were estimated in each group from 10 animals chosen at random. The ratio between the total volumes of the two cell systems was changed in the same direction as in the case of the B/A cell number ratio. Thus the A cell fraction of the total islet volume with the carbohydrate rich diet was $10.41 \pm 0.62$%, while the value with the carbohydrate poor diet was $14.64 \pm 0.77$%. The corresponding figures for the B cells were $83.39 \pm 0.77$% and $80.65 \pm 0.72$%.

The B/A cell number and volume ratios appear to be sensitive indicators of the reactions of the islets to changes in the carbohydrate content of the diet. Significant changes in the total islet volumes, as found by Tejning (41), were not observed in our animals. However the experiments are not really comparable because great individual variations exist, and thus only with a much larger number of animals and perhaps a longer dietary period might it have been possible to obtain decisive results in our experiments.

### *Diurnal Changes in the Function of the Islet Cells as Indicated by the Nuclear Size (VI)*

It is known that the glycogen content of the liver is subject to diurnal changes. The close relationship between the internal secretory activity of the pancreas and this function of the liver gives reason to

suppose that the glycogen rhythm may reflect rhythmical changes in the functional state of the islet cells.

The blood flow through an organ is in general a sensitive criterion of its functional state. Although many authors have interpreted an islet hyperemia as a sign of increased activity of the B cells, theoretically it might also be explained on the basis of a hyperactivity of the A cells when B cell function might be expected to be depressed. Preliminary results from our laboratory support the idea that hyperemia may exist with either augmented or depressed B cell function (13). It should be possible to estimate changes in the functional state of the A and B cells by autoradiographic studies of their uptake of different substances. Though the substances hitherto used were not suitable for such studies (4, 5), the introduction of the tritiated nucleic acid precursors thymidine and cytidine seems to offer great promise, and they are at present being tested in islet studies. It has been clearly shown both by other authors and in our experiments (12, 28, 29) that the size of the nuclei of the islet cells forms a simple and sensitive indicator for the functional state of the cells. The diurnal changes in the nuclear size were therefore recorded by caryometry. Nuclear enlargement accompanying a rise in the functional state of the islet cells seems to be a result not only of an increase in the nuclear water content but also of an increase in the protein content (39).

Altogether 60 adult male rats which had been allowed free access to food were studied. At the season of the experiment sunrise was at 5.17, and sunset at 18.08. Ten rats were killed by decapitation every 4th hour, beginning at midday. Special care was taken to carry out the histological treatment of the pancreas in a similar way throughout. With the help of an ocular screw micrometer the largest diameter and that at right angles to it were measured in the optically greatest cross section of the nuclei (magnification 1775×). In each animal 24 A cell nuclei from at least 3 islet section surfaces and 50 B cell nuclei from at least 5 islet section surfaces were measured. Twelve similar determinations in one animal yielded a random error of 2.04% for the A cells and 1.57% for the B cells. The mean size of the B cells displayed two maxima, one at 12 noon and the other at 12 midnight. In contrast to the midnight value, the midday value, corresponding to a nuclear surface area of $25.76 \pm 0.36\ \mu^2$, differed probably significantly ($P < 0.05$) from the immediately preceeding ($24.59 \pm 0.41\ \mu^2$), and succeeding ($24.55 \pm 0.40\ \mu^2$) values. The midnight value of $25.26 \pm 0.29\ \mu^2$ constituted on the other hand the top of a curve of successively rising and falling values. The sizes of the A cell nuclei, which tended to be higher during the later daylight hours, showed a significant decrease from the maximum value of $21.99 \pm 0.31 \mu^2$ at 8 p.m. to $20.76 \pm 0.18\ \mu^2$ at midnight ($P < 0.01$). The ratio between the nuclear surface areas of the B and A cells was at a prominent minimum

at 8 p.m., 1.130±0.013, followed by a highly significant rise to a maximum value of 1.217±0.011 at midnight ($P < 0.001$).

Whether this islet rhythm is primary and endogenous or secondary to extra-pancreatic factors affecting the blood sugar is not as yet possible to state. Since the rat is a nocturnal animal the midnight maximum in the nuclear size of the B cells may be explained by the fact that the main food intake takes place after dark. The low A cell value at the same time can be well interpreted as due to a post-alimentary hyperglycaemia. Holmgren (23) in his investigations of the diurnal changes in the liver glycogen found a maximum around 8 a.m., and a minimum around 4 p.m. Studies of glycogen stained liver sections from our rats seemed to indicate that these maxima and minima appeared earlier and corresponded better with the 4 hour period immediately previous. As however glycogen stained liver sections alone were not considered to give sufficient information of the changes in the liver glycogen, further discussion on the relation between these changes and the diurnal rhythm in the functional state of the islet cells will be deferred to another paper.

## C. The Effect of Protracted Administration of small doses of Alloxan on the Islet Organ in the Rat (VII)

Whether it is possible to obtain a B cell necrosis with diabetogenic doses of alloxan in the absence of extra-pancreatic factors has been studied previously (3, 19). Although it now seems to be generally accepted that alloxan exerts a direct effect on the B cells, the more detailed mechanism of this is not clear. It is not even certain that alloxan always inhibits or destroys the B cells. Thus in recent years several authors have put forward the view that the necrosis is always preceeded by an initial stimulatory phase, and that these stimulatory properties are completely dominant if the dose is small and repeated several times. Hughes (24), for example, summarized his observations on the cytological effects of small alloxan doses in the rat in the following way: 'if the dose is small and repeated daily for several days the number of islets in the gland seems to be greater, the average size of the islets is increased and the diameter of the B cells in the large islets also increases'.

In co-operation with C. Hellerström (20), it was shown that the increased nuclear size which has been reported after single diabetogenic alloxan doses does not necessarily imply a stimulating effect, as it probably resulted only from increased functional demands on the surviving B cells. The investigations have now been extended to include an analysis, carried out with quantitative histological methods,

of the effect of protracted administration of small doses of alloxan. As great importance has been attributed to the carbohydrate content of the diet in the hypothesis of an endogenous alloxan production (34), and an increase of the carbohydrate content of the diet is itself a stimulus for the islet tissue, the effect of the small doses of alloxan has been studied both with carbohydrate rich and carbohydrate poor diets.

The islet analyses have been carried out on altogether 56 young male rats, which were fed on a diet containing either 74% or 2% carbohydrate. In 27 animals alloxan was injected subcutaneously every 8th hour for 75 days, in a dose of 5 mg/kg body weight. The remaining rats received the same quantity of Sørensen's phosphate buffer alone (pH 5.8–5.9). The subcutaneous diabetogenic dose of the alloxan preparation used was in adult rats 140–160 mg/kg body weight.

The rats tolerated the protracted alloxan treatment well. At the time of killing no differences were found in the body weights or hemoglobin values of the blood between alloxan treated and control rats. The liver, kidneys, testes, thymus, adrenal glands, thyroid and hypophysis were weighed and examined microscopically. For the carbohydrate rich diet the alloxan treatment resulted in probable decrease in the weight of the adrenal gland, from $42.10 \pm 2.83$ mg to $34.10 \pm 2.07$ mg ($P < 0.05$), and in a significant decrease in the thymus weight, from $321.1 \pm 13.3$ mg to $268.4 \pm 12.1$ mg ($P < 0.01$). With the carbohydrate poor diet after alloxan treatment there was only a probable decrease in the weight of the kidneys from $2232.4 \pm 89.9$ mg to $1962.3 \pm 59.9$ ($P < 0.05$). No microscopical pathological changes were found in the above named organs as a result of the alloxan treatment. The most obvious finding in a qualitative microscopical examination of the islets was the absence of pronounced cytological changes. An islet analysis carried out with quantitative histological methods showed however that both the number of islets and the total islet volume had decreased after protracted alloxan treatment. For the carbohydrate rich diet the islet volume in the control animals was $1.679 \pm 0.115$ $mm^3$ compared with $1.348 \pm 0.090$ $mm^3$ after alloxan ($P < 0.05$). Corresponding figures for the carbohydrate poor diet were $1.628 \pm 0.133$ $mm^3$ and $1.198 \pm 0.067$ $mm^3$ ($P \approx 0.01$). This decrease could not be attributed to a lack of islets in any special size class because in spite of the alloxan treatment both the numerical and the volumetric distribution curves retained the form characteristic of the untreated rats.

The composition of the islets was markedly changed after the protracted alloxan treatment. The B/A cell number ratio thus decreased for the carbohydrate rich diet from $2.89 \pm 0.13$ to $2.01 \pm 0.07$ ($P < 0.001$), and for the carbohydrate poor diet, from $2.15 \pm 0.08$ to $1.61 \pm 0.04$ ($P < 0.001$). An investigation of how the B/A cell number ratio varied with the size of the islet section surfaces showed that the

relatively higher values for the small islet section surfaces which were found in the control animals tended to be evened out after alloxan administration. If the ratio was expressed as a percentage of the original differential cell count value, the values in the control animals on the carbohydrate rich diet were for example $139.6 \pm 9.4\%$ for the smallest islet section surfaces containing $< 16$ cells, compared with $106.6 \pm 4.5\%$ for the largest islet section surfaces ($P < 0.01$). After treatment with alloxan the corresponding values were $119.2 \pm 10.5\%$ and $102.3 \pm 2.5\%$ ($P > 0.05$).

By combining in each rat the values for the individual mean cell volumes of the A and B cells, the relative volume of the residual islet tissue (i.e. that which does not consist of A or B cells), the total islet volume and the B/A cell number ratio, the total volumes of the A and B cells have been calculated separately in 10 animals chosen at random from each of the four experimental groups. For the total A cell volume no significant differences were recorded between the alloxan treated animals and the corresponding control group. The total B cell volume was on the other hand probably lower in the alloxan treated groups. For the carbohydrate rich diet it was $1.031 \pm 0.073$ mm$^3$ compared with $1.380 \pm 0.129$ mm$^3$ for the control animals ($P < 0.05$); the corresponding values for the carbohydrate poor diet were $0.990 \pm 0.054$ mm$^3$ and $1.383 \pm 0.160$ mm$^3$ ($P < 0.05$). In a comparison of the compositions of the islet volume in the different groups, characteristic changes were seen. The protracted administration of alloxan thus resulted in a significant decrease in the percentage of the B cells. While the B cell contribution in the carbohydrate rich diet was $83.39 \pm 0.77\%$, a value of $78.90 \pm 0.36\%$ ($P < 0.001$) was obtained after treatment with alloxan. The corresponding values for the carbohydrate poor diet were $80.65 \pm 0.72\%$ and $77.44 \pm 0.53\%$ ($P < 0.01$).

The results obtained provide strong evidence against the hypothesis that alloxan can exert a stimulatory effect on the B cells of the islets, and are therefore in good accord with our earlier experimental results (20). The possibility can however still not be entirely excluded that the B cell depression by small alloxan doses might be converted into a stimulation by altering the experimental conditions in some way, e.g. by changing the interval between the doses.

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BO HELLMAN: *Quantitative Studies on the Islets of Langerhans*

## ERRATA

*Paper I.* Page 338, line 24. For 'vertical resolution' read 'depth of field'.

Page 341, line 32. For 'The non-silver method was used in the present investigation. The technique used phloxine' read 'The non-silver treated sections were stained with chrome-hematoxylin phloxine' . . .

*Paper II.* Page 9. Last sentence of first paragraph. For 'As the values obtained according to formula (2) . . . total islet volume of the rat' read 'The values obtained according to formula (2) have only been used for calculation of the correlation coefficients in the different age groups, while the other values were calculated from the actual size distribution of the islets'.